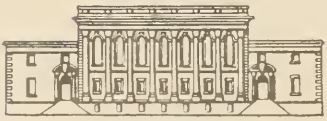


Isolation of Chloroplast DNA from *Nicotiana tabacum* for use in investigations of phylogeny.

Margaret Enochs
April 1983

ABSTRACT

The purpose of this project was to determine the phylogenetic relationships in the genus Nicotiana by electrophoretic analysis of chloroplast DNA treated with restriction endonucleases. A great deal of time was devoted to the development of a procedure for isolating chloroplasts and chloroplast DNA using only low speed centrifugation. So much time was devoted to developing the procedure that it was applied to only one species, N. tabacum.

One banding pattern was obtained repeatedly under a variety of conditions. This consisted of one band near the top of the gel. A few other patterns were obtained unpredictably. At this point the isolated cases of banding patterns which had more than one band are inexplicable. The one band obtained in most cases was probably native chloroplast DNA.

If the conditions of electrophoresis could be altered to reveal the fragmentation of DNA due to restriction digestion, then the identity of the DNA isolated could be determined. Confirmation of the DNA as chloroplast DNA would prove that the procedure given in Materials and Methods could be a very valuable tool for the investigation of phylogenetic relationships in plants.

LITERATURE REVIEW

The evolutionary relationships between the species of some plant families have not been completely or conclusively determined. The methods used to study this problem range from examination of gross morphology to chemical analysis of DNA and proteins. Advantages of the procedure given in Materials and Methods are its speed, the relative ease of its use, and a specificity which shows differences between closely related species (1,16).

The treatment of DNA with type II restriction endonucleases produces specific fragmentation along the entire length of the DNA. Electrophoresis of these fragments results in a banding pattern which is specific for the type of DNA and the restriction enzyme used (11,16). Analysis of the restriction pattern of the chloroplast DNA could help establish the female parentage of a species. Chloroplast DNA is smaller and less complex than nuclear DNA and thus is easier to work with.

Plants of the Nicotiana genus were used for this study because they are readily available, are easily grown, and have been used in studies of the function of chloroplast DNA. It was necessary to begin by working out procedures for the isolation of chloroplasts and chloroplast DNA which required only low speed centrifugation. Previously published procedures, without exception, used ultracentrifugation (11, 12, 16, 18). The use of low speed centrifugation would reduce the stress on the DNA and could make the yields of DNA higher than when ultracentrifuged, not to mention reducing the cost and difficulty of the procedure. The purity of the DNA isolated was not completely confirmed, but the results support the plausibility of the attempt to develop such a procedure.

CHLOROPLAST DNA

In 1908 the variegation of Nicotiana tabacum leaves was shown to be inherited from the female parent alone (19). This was the first indication of the presence of genetic material in the cytoplasm, since the cytoplasm is also inherited only from the female parent. The cytoplasmic genetic material is found in chloroplasts and mitochondria. Chloroplast DNA (cpDNA) from different species is very similar, indicating that the evolution of chloroplasts has been conservative (1). This is at least in part due to the presence of multiple copies of the chloroplast DNA; it is unlikely that a single mutation would ever show up (1,8).

CpDNA has a molecular weight of $11.4 \times 10^7 \pm 1 \times 10^7$ daltons and its density in a CsCl gradient is 1.702, compared to 1.696 for nuclear DNA. CpDNA has no 5-methyl cytosine which is found in nuclear DNA (15). The histones associated with nuclear DNA are absent from cpDNA. The absence of these proteins means that there is less control on the transcription of chloroplast DNA than there is on nuclear DNA (14). Some cpDNA fragments are circular and within a species the base sequence of the circular DNA is a constant (2).

The chloroplast has its own protein making machinery, including ribosomes. Chloroplast DNA polymerase makes cpDNA-like DNA *in vitro* and has different properties from the nuclear enzyme (12). There is also a unique chloroplast RNA polymerase, which shares some properties with the nuclear enzyme. They both require the presence of Mg++ and all four ribonucleoside triphosphates to work and both are inhibited by actinomycin D (14).

Chloroplast RNA is from 4 to 30S in size and includes all types of

RNAs (15). Nuclear DNA hybridizes to ribosomal RNA from both chloroplast (70S) and cytoplasmic (80S) ribosomes, but in different places on the nuclear chromosome (13). There is one cistron for the 70S ribosomal RNA on the nuclear genome (15). Chloroplast DNA hybridizes only to cpRNA (13). On the cpDNA there are eight cistrons for ribosomal RNA, four each for the two components of the 70S ribosome. In comparison, there are 2,000 cistrons on the nuclear DNA for the cytoplasmic ribosomal RNA. There are more cistrons for the cytoplasmic ribosomal RNA both because there is need for greater protein-making capacity in the cytoplasm than in the chloroplast and because the nuclear DNA is more repressed from transcription than is the chloroplast DNA (13).

The nuclear and chloroplast genomes cooperate in the control of the construction and function of the chloroplast. The nuclear DNA codes for: ferredoxin (10), three proteins of the 70S ribosome (Borque, Wildman as cited in 9), the protein of the Photosystem II chlorophyll-protein complex (9) and the small subunit of ribulose 1,5-bisphosphate carboxylase (8). The cpDNA codes for some of the activity of Photosystem II, the protein of the Photosystem I chlorophyll-protein complex (9), and for the large subunit of RuBP carboxylase (8). Both chromosomes may also contain genes not yet discovered for chloroplast structure or function.

Theories of the Origin of Some Nicotiana Species

There are twenty species of *Nicotiana* in Australia and forty in the Americas (19). The genus probably originated in South America and migrated to its present location (5). Many of the modern species arose from

hybridization of preexisting species (Goodspeed, Burbidge, as cited in 19). The haploid numbers of Nicotiana species are, in most cases, multiples of 6 or 12 and anaylsis of these numbers gives insight into the hybridizations which must have taken place in order for modern species to arise. (See Table 1).

There are examples of speciation by hybridization. This sort of speciation has been shown to occur in the laboratory; creation of N. digluta occurred when N. glutinosa and N. tabacum were crossed (Clausin, Goodspeed, 1925, as cited in 19). As another example, tryptic digest of N. digluta RuBP carboxylase and separation of the peptides by chromatography show that the small subunit has two peptides identical to N. glutinosa and two identical to N. tabacum. The large subunit peptides are identical to the large subunit peptides of N. glutinosa (19). This is precisely the similarity between the proteins which would be expected if N. digluta were indeed the progeny of N. glutinosa and N. tabacum.

Synthetic hybrids of modern N. sylvestris and members of the Tomentosae section are similar to N. tabacum, indicating that N. sylvestris and a Tomentosae might be the parents of N. tabacum. The morphology of the hypothesized parent plants is similar to the offspring. The karytoype of N.tabacum could be a simple combination of the karyotypes of N. sylvestris and the Tomentosae (5).

All investigations indicate that N. sylvestris must have been the female parent of N. tabacum. The male parent is a matter of some contention. Electrofocussing of RuBP carboxylase indicates that either N. otophora or N.tomentosiformis was the paternal parent (6, Gray as cited in 7). The present geographic location of the species would make it impossible for

N. tomentosiformis to be the male parent, as it is not found near N. sylvestris. N. otophora is found near N. sylvestris (7, 5). However, the modern distribution of plants does not necessarily indicate the ancient distribution.

Electrophoresis of peroxidase, catalase and six other enzymes indicate that N. tomentosiformis is the paternal parent of N. tabacum (Sheen as cited in 7). Chromatography of chymotryptic digests of the RuBP carboxylase of the hypothetical parents indicates that N. sylvestris was the female and that N. tomentosiformis was the male which hybridized (7).

Table I (5)

Section	Species	Haploid Number
Paniculatae	N. benvidesii	12
	N. cordifolia	12
	N. glauca	12
	N. knightiana	12
	N. paniculata	12
	N. raimondii	12
	N. solanifolia	12
Thyrsiflorae	N. thyrsiflora	12
Rusicae	N. rustica	24
Tomentosae	N. glutinosa	12
	N. otophora	12
	N. stechellii	12
	N. tomentosa	12
	N. tomentosiformis	12
Genuinae	N. tabacum	24
Undulatae	N. undulata	12
	N. wigandioides	12
	N. arentsii	24
Trigonophyllae	N. palmeri	12
	N. trigonophylla	12
Alatae	N. alata	9
	N. bonariensis	9
	N. forgetiana	9
	N. langsdorffii	9
	N. longiflora	10
	N. plumbaginifolia	10
	N. sylvestris	12
Repandae	N. nesophila	24
	N. repanda	24
	N. stocktonii	24
Noctiflorae	N. acaulis	12
	N. noctiflora	12
	N. petunioides	12
Acuminatae	N. acuminata	12
	N. attenuata	12
	N. corymbosa	12
	N. linearis	12
	N. miersii	12
	N. pauciflora	12
	N. spegazzinii	12
Bigelovianae	N. bigelovii	24
	N. clevelandii	24
Nudicaules	N. nudicaulis	24
Sauveolentes	N. exigua	16
	N. maritima	16
	N. suaveolens	16
	N. velutina	16

Table I (cont.)

Section	Species	Haploid Number
	N. gossei	18
	N. benthamiana	19
	N. excelsior	19
	N. goodspeedii	20
	N. megalosiphon	20
	N. rotundiflora	22
	N. debneyi	24
	N. fragrans	24
	N. occidentalis	24

Use of Restriction Digests to Determine Phylogeny

There remains contention over the male parentage of N. tabacum. The geographic location indicates that N. otophora is the male parent but many of the chemical tests indicate that N. tomentosiformis is the parent. Investigation of the parentage of all Nicotiana species could be done by electrophoresis of specific restriction endonuclease digests of both the chloroplast and nuclear DNA. The chloroplast DNA would reveal only the female parentage, but once that parent is established, the identification of the male parent is much easier. The female parent's contribution to the nuclear DNA could be established and eliminated from consideration of the male parent's contribution.

There is a correlation between the similarity of cpDNA restriction digests of two species of plants and the ease with which those plants are hybridized (1). Therefore, members of the Nicotiana species, which hybridize easily, are well suited for analysis by restriction digest. The banding patterns can be expected to be similar enough to be easily compared.

The similarity between the restriction patterns of several species of Nicotiana has already been established. The Eco RI digestion pattern of the cpDNA of three American species (N. tabacum, N. bonariensis , N. langsdorfii) was determined. There were two bands which N. tabacum did not have but the others did. The patterns for three Australian species (N. excelsior, N. sauveolens, N. gossei) were similarly determined. The patterns for the Australian species were identical. There was a difference of only five bands between the American and Australian species, excepting the two bands missing from N. tabacum that the other American species had (1). Another experimenter found two bands difference between N. tabacum and N. glauca and two bands difference between N. tabacum and N. rustica (17). The other species of the genus could be expected to show the same kinds of similarities, particularly in light of the similarities between the Australian and American species. The similarities indicate not only the close relatedness of the species but the conservative evolution of the choroplasts in the genus.

Specific restriction patterns have been used to determine relationships between other plants, and also to identify other cytoplasmic DNA. The Eco RI restriction patterns of cpDNA have been used to confirm the phylogeny of diploid, tetraploid and hexaploid wheats. The species involved were Triticum monococcum, T. dicoccum, T. timopheevi, T. aestivum, Aegilops squarrosa, and Ae. speloides. It is notable that the scheme confirmed was partially based on the ability of these species to form hybrids (18). Specific endonuclease digestion has also been used to characterize plasmid DNA (16) and phage DNA (11).

MATERIALS AND METHODS

Plant Material: Nicotiana tabacum plants, variety Wisconsin 38 and an unidentified variety obtained from the U. S. Department of Agriculture, were grown in a mixture of perlite and potting soil under intense light for 2 months. Immediately before harvesting the plants were left in darkness for 5-7 days at 16C. Approximately 200g of leaves were harvested and washed in dilute Ivory detergent, rinsed in tap water, distilled water and glass distilled water. Sometimes approximately 400g of leaves were used, depending on the health of the plants. Washing and grinding of the leaves were carried out in a cold room. The plant products were kept cold until the chloroplasts were lysed.

Chloroplast Isolation: One liter of Kool's Buffer A (50 mM Tris/HCl, 0.35M sucrose, 7mM Na2EDTA, 5mM 2-mercaptoethanol, pH 8) (12), containing 0.1% bovine serum albumin (BSA) was added to every 200g of leaves and ground three times for 5 seconds at high speed in a Osterizer blender. The mixture was filtered through four layers of cheesecloth and two layers of Miracloth or eight layers of cheesecloth and two layers of sieve cloth (420uM mesh).

The filtrate was centrifuged for 10 minutes at 1000xg. The pellet was resuspended in 40 ml of Kool's Buffer A without BSA. This suspension was then layered onto a sucrose step gradient prepared by layering 5 ml each of 15%, 20%, 30%, and 45% sucrose in Kool's Buffer A in a 50 ml centrifuge tube. This was centrifuged for 50 minutes at 1000xg. The green layer between the 30% and 45% steps, as well as some of the dark green pellet in the 45% steps, were collected. The chloroplast layer was then suspended in 5ml of Kool's

Buffer B (50mM Tris/HCl, 20mM EDTA, pH8) (12) and centrifuged for 5 minutes at 4800xg.

DNA Isolation: The pellet from the 4800xg centrifugation was resuspended in 0.5ml of 0.2% DNase in water and incubated for 20 minutes at room temperature. This suspension was centrifuged for 5 minutes at 4800xg. The pellet was resuspended in 5ml of Kool's Buffer B, and was again centrifuged for 5 minutes at 4800xg. The final pellet was resuspended in 5ml of Kool's Buffer B.

The chloroplast suspension was then mixed with 1ml of 18% Sarkosyl in water and incubated for 20 minutes at room temperature. One milliliter of 0.04% Protease in water was added and the suspension was incubated 2 hours to overnight.

The suspension was extracted with phenol three times: approximately 5ml of phenol were mixed with the crude DNA solution and then centrifuged at high speed in an IEC clinical centrifuge. The aqueous layer was collected and the extraction was repeated.

One-tenth volume of chilled 3M NaAc and 2.5 volumes of chilled 95% alcohol were added to the aqueous layer to precipitate the DNA. The DNA was allowed to dry in air in front of a fluorescent lamp after removal of the excess liquid and was then brought up in 100ul of water. Spectrophotometric analysis was used to determine the concentration of DNA; A260 = 25 if the concentration of DNA is 1mg/ml (4).

Hill Reaction: Two ml of dilute chloroplast suspension (1ml concentrated chloroplast suspension and 9ml Hill Reaction Buffer) were added to 3ml of Hill Reaction Buffer (0.2M KH_2PO_4, 0.2M Na_2HPO_4, 0.5M sucrose) and 1ml of 2,6-dichlorophenolindophenol (DPIP). Reduction of the DPIP was followed

by determining A620 at 10 minute intervals.

The condition of the chloroplasts was tested by removing aliquots of the chloroplast suspension at different times during the isolation and assaying the aliquots for Hill Reaction activity at intervals for the duration of the isolation. (See Diag. A). The aliquots were active at all stages. The aliquots were also inspected under the phase microscope and were found to contain a high percentage of intact chloroplasts throughout the isolation.

Electrophoresis: The DNA was treated with Eco RI and electrophoresed. Comparison of the banding patterns with previously published patterns (1,17) could have been used to determine the purity of the DNA.

Three to twenty-five ul of DNA solution, 3ul of Eco RI and 5ul of Endo R-Reaction Buffer (500mM Tris/HCl, 50mM MgCl2, 50 mM $CaCl_2$, 20mM 2-mercaptoethanol, pH 7.4) (3) were brought up to 50 ul with water. This solution was incubated for 30 minutes at 37C. The enzyme action was stopped with a Stop solution (0.07% Na2EDTA, 10% Sodium Dodecyl Sulfate (SDS), 0.1% Bromphenol blue, 10% glycerol in water). One per cent agarose gels were prepared by mixing agarose with 1 part concentrated Electrophoresis Buffer (0.4M Tris/HCl, 0.2M NaAc, 20mM Na2EDTA, pH 7.9) (3) and 9 parts water. The solution was heated to boiling and poured into tubes 0.5cm in diameter and 13cm long. The gels were fitted into a BioRad 150A gel electrophoresis apparatus and the circuit was completed with Electrophoresis Running Buffer (1 part Concentrated Electrophoresis Buffer and 9 parts water) (3).

The DNA solution was underlayered onto the gels. The gels were then

Diagram A

Step of Isolation	Aliquot removed	Hill Reaction #1 init. A620 / final A620 — done when A1.1 was removed	Hill Reaction #2 init. A620 / final A620 — done when A1.2 was removed	Hill Reaction #3 init. A620 / final A620 — done when A1.3 was removed	Hill Reaction #4 init. A620 / final A620 — done when A1.4 was removed
Leaves Chopped					
Leaves Filtered	A1.1	0.13 0.01	0.42 0.07	0.46 0.02	0.62 0.195
Centrifuge 10'x1000xg					
Resuspend pellet in Kool's BufferA	A1.2		0.46 0.09	0.6 0.065	0.58 0.07
Centrifuge 10'x1000xg					
Resuspend pellet in Kool's Buffer A	A1.3			1.85 0.38	0.45 0.05
Gradient					
Collect green layer	A1.4				0.58 0.06

run at 100v until the tracking dye was approximately 3cm from the end of the gel; this usually required 1-1½ hours.

The gels were removed from the electrophoresis apparatus and stained with 0.001% ethidium bromide in water. The gels were examined under UV light and the distance each band had travelled was recorded.

The technique described above was adapted from BRL Concepkit 1 (3) with the following exceptions:

1) Tube gels 0.5cm in diameter and 13cm in length were used instead of slab gels.

2) The ethidium bromide was at a concentration of 0.001% rather than the unidentified concentration provided with the kit.

3) The Stop solution was created and substituted for the Endo R-Stop solution provided with the kit.

Development of Methods: The initial experiments which were used to determine the method described were done on geranium leaves.

The sucrose concentrations used in the gradient were selected by centrifuging a chloroplast solution through 50%, 30%, 15%, 50% sucrose solutions and microscopically examining the pellets. The 50% solution had the only pure chloroplast pellet, so the gradient used was 10%, 15%, 30%, 50%. The top two steps were changed to 15% and 20% when it was observed that the chloroplasts entered the top two steps immediately upon being loaded onto the gradient. The lowest step was changed to 45% to compensate for the sucrose already present in the buffer used to make the gradient.

The green layer removed from between the 30% and the 50% steps, as well as all the pellets, were viscous and lumpy. A tissue homogenizer was used to resuspend the pellets in order to prevent the lumps from interfering

with subsequent centrifugations. This later was considered to put too much stress on the chloroplasts and the DNA, so gentle brushing with a paintbrush was substituted.

Unless stated otherwise, all centrifugations were carried out in plastic centrifuge tubes or in glass tubes treated with Prosil-28 according to the instructions enclosed with the chemical. 1000xg centrifugations were achieved using a TH-4 rotor on a Beckman TJ-6 centrifuge. 4800xg centrifugations achieved using a TA-24 rotor on the TJ-6 centrifuge. Spectrophotometric analysis of Hill Reaction activity was performed using a Bausch and Lomb Spectronic 20. The concentration of DNA was found using a Perkin-Elmer Hitachi 200 spectrophotometer.

Chemicals and materials were acquired from the following sources: DNase I, sieve cloth, Sarkosyl, Protease K, Eco RI, and ethidium bromide from Sigma Chemical Company; phenol and Endo R-Reaction Buffer from Bethesda Research Laboratories, SDS and agarose from BioRad and Prosil-28 from PCR Research Chemicals.

RESULTS

The first three attempts at DNA isolation produced no precipitate when the DNA solution was treated with ethanol. In the first two attempts, no DNase treatment was used. In the third attempt, the DNase was used but it was not washed out of the chloroplast solution after the incubation.

When a washing (centrifugation followed by resuspension) was introduced after the DNase treatment and gloves were worn throughout the isolation of chloroplasts from 400g of not-very-healthy leaves, a precipitate of DNA was obtained (DNA Ia). The DNA was centrifuged (1 hour x 1000xg) and brought up to 100ul of water. The DNA was electrophoresed both with and without prior treatment with Eco RI (from BRL Concepkit I). Fluorescent bands and fluorescence at the tops of the gels were observed. No difference was seen between the DNA Ia which had been treated with Eco RI and the DNA Ia which had not been restricted. (See Dia. B). DNA Ia was restricted and electrophoresed with a new batch of Eco RI (Sigma). The gels showed a fluorescent band which was different from the one obtained with the old batch of enzyme, and fluorescence at the top of the gel. (See Diag. C). Varying the amount of enzyme used (from 1-3ul of Eco RI to 10 ul of DNA solution) did not change the banding of the gel. Drastic reduction of the amount of DNA Ia used produced one band on the gel, at approximately 1.4cm from the top of the gel when lul of DNA was restricted with 3ul of Eco RI, and at approximately 0.8cm from the top of the gel when 2, 3, or 6ul of DNA were restricted by 3ul of Eco RI. (See Diag. D).

The effect of an expansion of the incubation time was tested. Two ul of DNA Ia were incubated with 3 ul of Eco RI for 1 hour and for 10 hours before electrophoresis. After one hour of incubation

CENTIMETERS

12 10 8 6 4 2 0

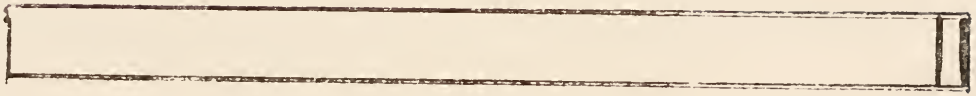
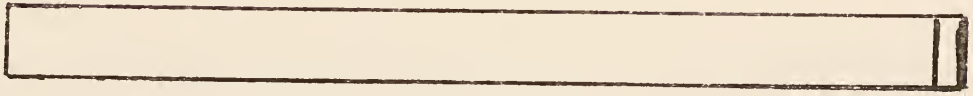
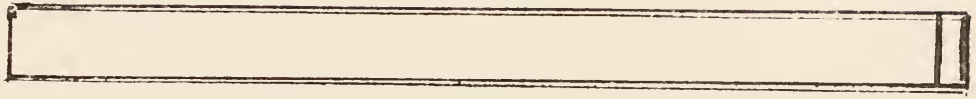
DNA	ECO RI	
Ia / 10ul	5ul	
Ia / 10ul	5ul	
Ia / 10ul	none	
Ia / 10ul	none	

DIAGRAM B

CENTIMETERS

DNA ECO RI

Ia 10ul 5ul

Ia 10ul 5ul

DIAGRAM C

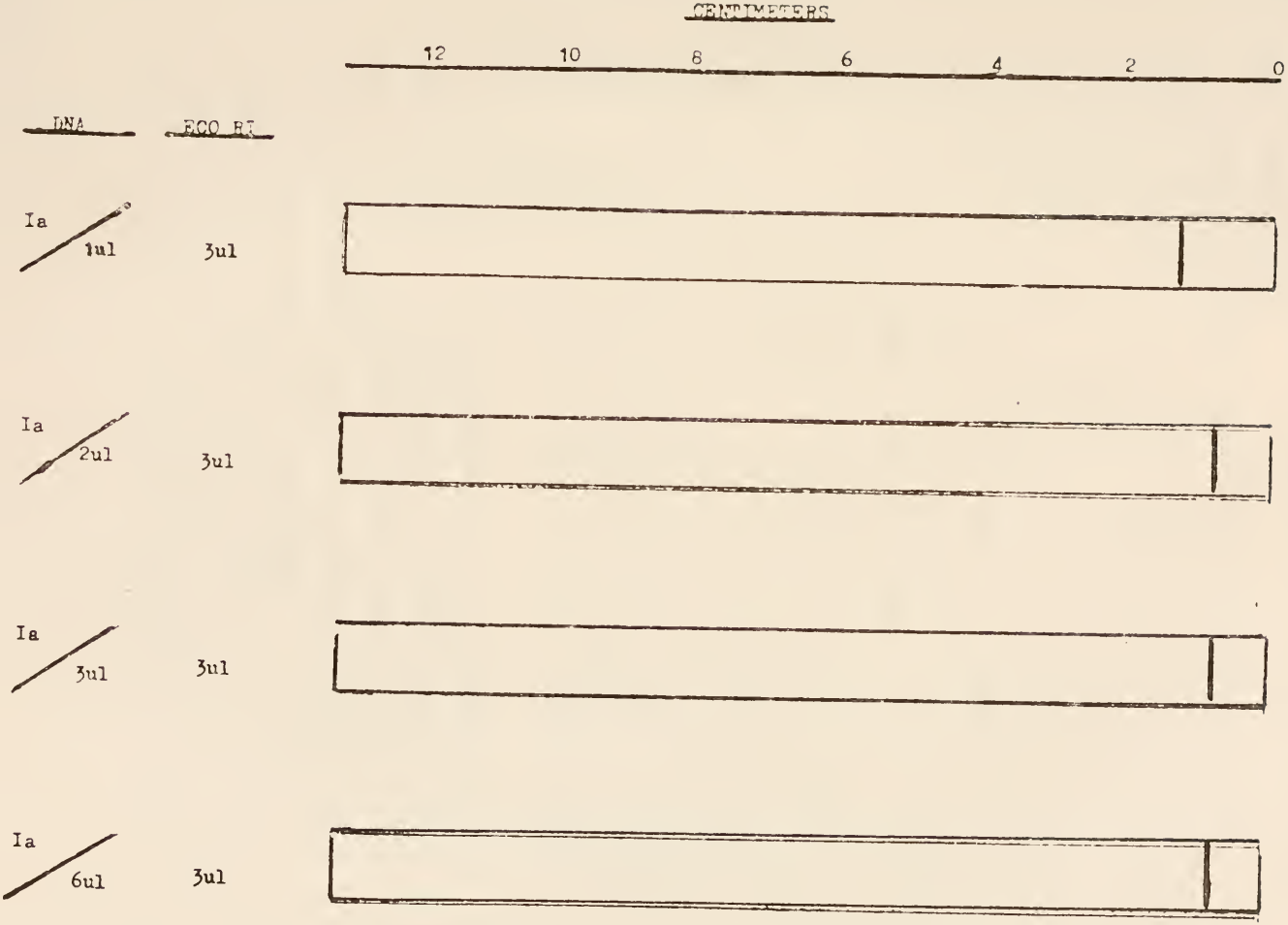

CENTIMETERS
12
10
8
6
4
2
0
DNA
ECO RI
Ia
1ul
3ul
Ia
2ul
3ul
Ia
3ul
3ul
Ia
6ul
3ul

DIAGRAM D

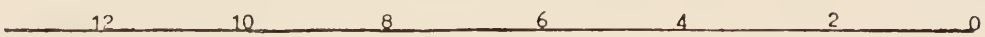
electrophoresis gave a band at approximately 12.5 cm from the top of the gel. (See Diag. E).

The DNA was reprecipitated with NaAc and alcohol as previously described following the detection of the odor of phenol in the DNA solution. It was then dried and brought up in 100ul of water (this became DNA Ib). Electrophoresis of the native DNA Ib (1, 2, 3ul) gave two bands on the gels, at approximately 0.7 and 1.1cm from the top of the gel. Repetition of the electrophoresis with different amounts of native DNA (4, 6, 8, 10, 15ul) gave a band at approximately 1.1cm from the top of the gel, the same band which appeared on almost every gel. (See Diags. F and G).

Another isolation of DNA was performed using exactly the same procedure as the previous isolation, except that the DNA was allowed to dry before being brought up in water. Electrophoresis of various concentrations of DNA II (3, 5, 7, 10, 15, 35, 35, 40 ul of DNA II solution with 3 ul Eco RI) resulted in confusing banding patterns: the same concentrations of DNA did not give the same patterns when repeated at a different time; concentrations which did not enter the gels in some cases did in others. (See Diags. H-J).

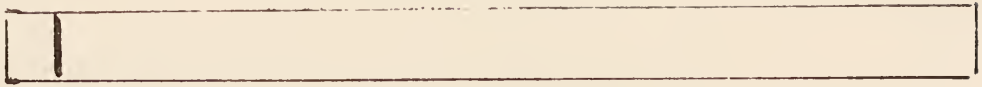
Another isolation was done using 200g of very healthy leaves. The DNA III resulting from the isolation and calf thymus DNA (BRL Concepkit I) were used to test the activity of the DNase. The A260s of solutions of 5ul DNA III and 1mg DNase, and of 5ul of calf thymus DNA and 1mg DNase, in buffer, were followed on the spectrophotometer. In 20 minutes, the incubation time used in the procedure, the A260 of the calf thymus DNA was reduced from 0.039 to 0.036 (on a scale of 0-1) and the A260 of the DNA III was reduced from 0.063 to 0.035. The DNase degraded DNA III

CENTIMETERS



DNA EcoRI

Ia
2ul 2ul
incubated for 1 hour



Ia
2ul 2ul
incubated for 10 hours

DIAGRAM E

CENTIMETERS

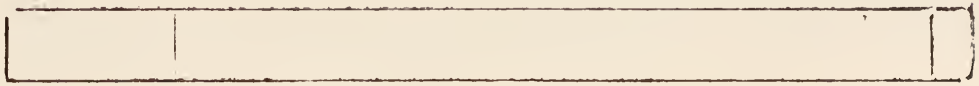
DNA	ECO RI	
Ib / 2ul	none	
Ib / 2ul	none	

DIAGRAM F

CENTIMETERS

12 10 8 6 4 2 0

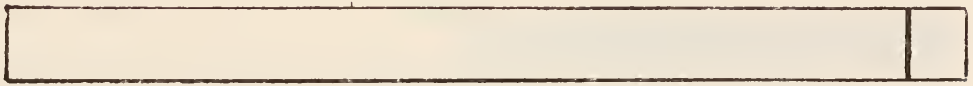
DNA	ECO RI	
Ib / 4ul	none	
Ib / 6ul	none	
Ib / 8ul	none	
Ib / 10ul	none	
Ib / 15ul	none	

DIAGRAM G

CENTIMETERS

12 10 8 6 4 2 0

DNA	ECO RI
II / 3ul	none
II / 5ul	none
II / 7ul	none
II / 10ul	none
II / 15ul	none

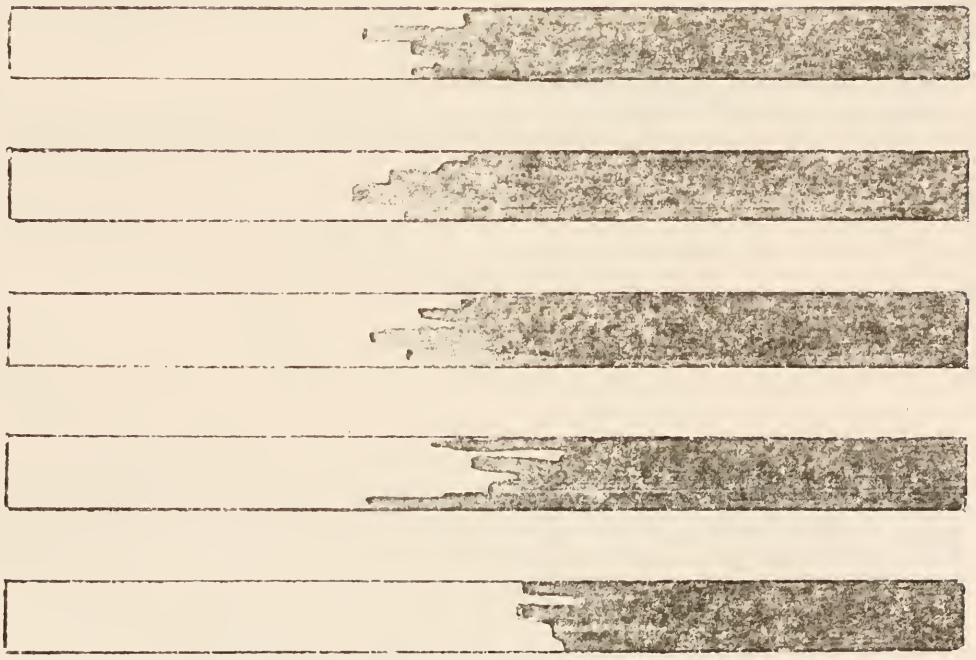


DIAGRAM H

CENTIMETERS

DNA ECO RI

II / 15ul 3ul

II / 25ul 3ul

DIAGRAM I

CENTIMETERS

12 10 8 6 4 2 0

DNA ECO RI

II / 25ul 3ul

II / 35ul 3ul

DIAGRAM I

further than it did the calf thymus DNA, but whether this was a sufficient fragmentation to prevent the precipitation of DNA is debatable. (See Diag. K).

The concentration of agarose was reduced from 2% to 1% in order to eliminate the problem of DNA not entering the gels. Electrophoresis of DNA III restricted with Eco RI was done. There was fluorescence at the tops of the gels, but no bands.

An isolation was done with 200g of healthy leaves. The chloroplast suspension was divided. Only half of the suspension was treated with DNase, and the other half was not treated with DNase; otherwise the procedures used were identical. Electrophoresis of the DNA IVa (DNase treated) and DNA IVb (not DNase treated) after treatment with Eco RI gave very similar smears on the gels. (See Diag. L).

Diagram K

Tube:	Contents: DNA	DNase	A260 at 0 minutes	A260 at 20 minutes
1	N. tabacum 5 ·1	1mg	0.063	0.035
2	Calf thymus 5ul	1mg	0.039	0.036

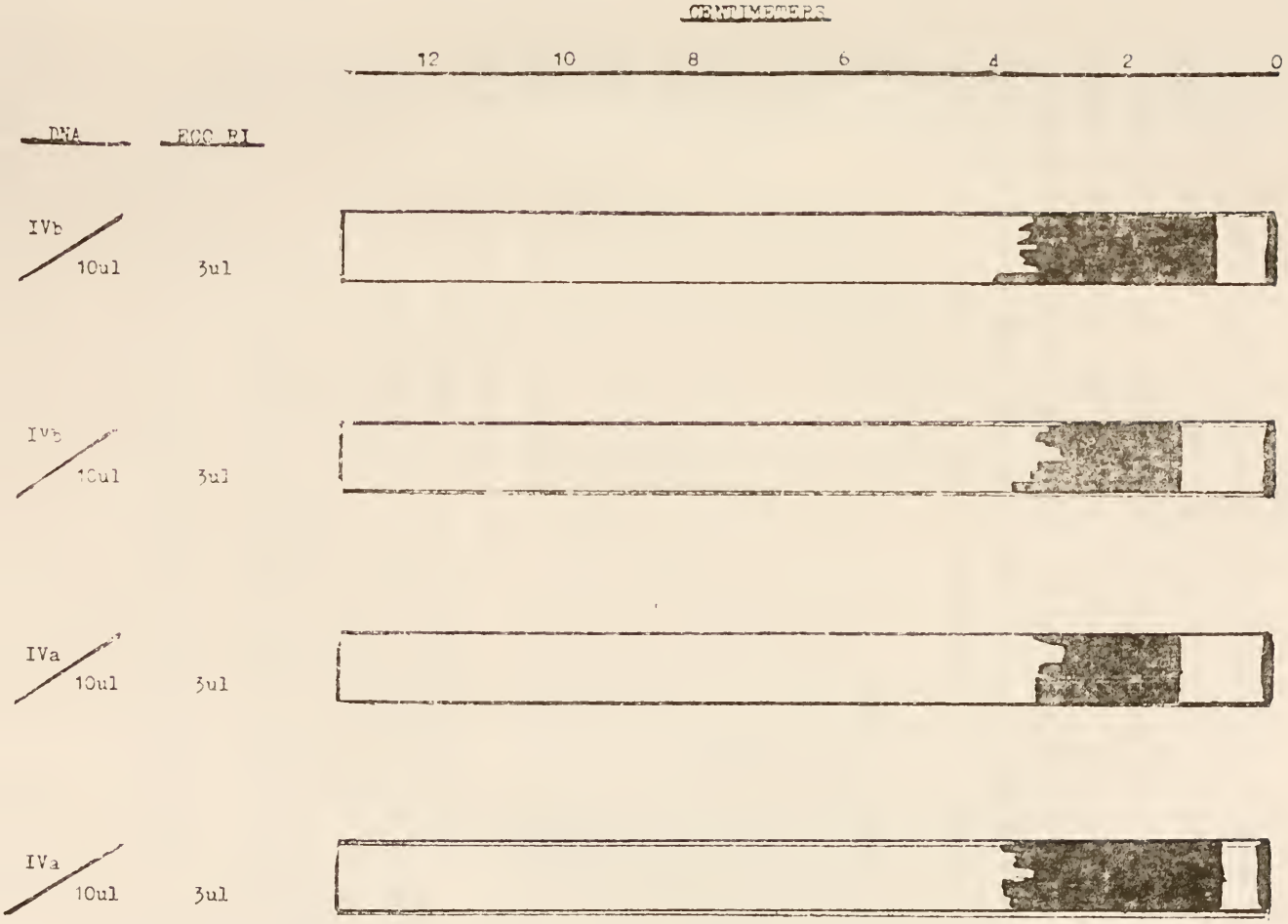

CENTIMETERS
12
10
8
6
4
2
0
DNA
ECO RI
IVb
10ul
3ul
IVb
10ul
3ul
IVa
10ul
3ul
IVa
10ul
3ul

DIAGRAM 1.

DISCUSSION

The two single factors which probably exerted the greatest influence over the results were the time necessary to perform the isolations and shearing stresses on the chloroplasts and DNA. The time necessary to perform the isolation decreased steadily with repetition. This was probably the reason the first few attempts to precipitate DNA failed, though conta minating DNases from skin, present during the isolation and during the plants' growth, may also have lysed the DNA into such small pieces that it would not precipitate.

The third isolation attempt may have failed to precipitate DNA because the DNase treatment was not followed by a washing and so the DNase might have degraded the DNA as soon as the DNA was released from the chloroplast. The washing was not used because the protocol called for Sarkosyl and Protease treatments immediately after the DNase treatment. There was some chance that the DNase might have been denatured by the Protease and Sarkosyl, but with the perversity common to all macromolecules, the DNase that needed great care to prevent its denaturation prior to its use was most difficult to get rid of once it had served its purpose.

As soon as a certain number of steps were taken to protect the chloroplasts and the DNA, such as wearing gloves throughout the chloroplast and DNA isolations and care of the plants, and washing after DNase treatment, and the expertise of the experimenter increased, the DNA precipitated. There was no problem precipitating the DNA after this and four successful isolations were done.

The DNA from the first isolation (DNA Ia) gave the same banding

pattern when the DNA was restricted with Eco RI as when no enzyme was used. (See Diags. B, D). The one band which was seen also occurred when DNA from other isolations was electrophoresed. The band, which was always between 0.5cm and 1.8cm, was probably native chloroplast DNA. No banding pattern which could have been the result of restriction was ever seen; no more than two bands were ever seen. It is likely that the DNA solutions used were too concentrated for the amount of restriction enzyme used, and it is possible that if more enzyme were used a restriction pattern would be seen. The fact that the pattern obtained after an hour of incubation had only one band reinforces the idea that a more complete pattern might be obtained with a large amount of Eco RI and a short incubation time. (See Diag. E). This has not been investigated thoroughly.

DNA Ia was reprecipitated and the result was DNA Ib, which was substantially different from its precursor. Electrophoresis of unrestricted DNA Ib resulted in two bands, one of which was much farther from the top of the gels than the bands obtained with DNA Ia (See Diags. F, G). This band was far from the top due to the small size of the DNA. Once the DNA had dried it was very difficult to resuspend and it was exposed to room temperature for long periods of time, as well as to a great deal of mixing. Most DNA is not stable at room temperature and the mixing may have sheared the DNA, both causing small pieces of DNA to go into solution.

Another isolation was done and the DNA (DNA II) was immediately dried and then resuspended, with the difficulty in resuspension mentioned before. The first electrophoresis of DNA II gave smears of fluorescence on the gels, indicating that DNA II was badly sheared and that restriction by

5ul of Eco RI made the situation worse or, perhaps, that there was nuclear DNA in the solution. (See Diag. H). When a 15ul sample of DNA II was restricted with a 3ul sample of Eco RI and electrophoresed, one band was seen in approximately the place that DNA Ia banded when restricted with a large amount of enzyme. (See Diag. I).

DNA III was electrophoresed using 1% gels, whereas 2% had been used before. When DNA III was restricted with small amounts of Eco RI and electrophoresed, the DNA stayed at the top of the gels. Too much DNA III was used for the amount of Eco RI used. That an amount of DNA III as small as 5ul was too large for 3ul of Eco RI to restrict into pieces small enough to enter a soft gel means that either the DNA III solution was very concentrated or that the unrestricted DNA III was very large.

Electrophoresis of both DNA IVa and DNA IVb after restriction with a proportionately small amount of Eco RI gave very similar patterns. Consequently, the DNA was probably not affected by the DNase treatment. There could be two reasons for this: the DNase might not have been active enough to degrade all the nuclear DNA into insignificant bits, and the nuclear DNA showed up on the gels, or, the chloroplast DNA which precipitated was so sheared that it would not form a tight band.

Apparently two kinds of DNA have been isolated. The problem remains to determine the sources of the two DNAs. The assumption can be made that they are nuclear and chloroplast DNAs. There is reason to think that DNA which banded at about 0.5 to 1.8cm is native (unrestricted) chloroplast DNA (17). It showed up as native cpDNA when it was untreated or when the cpDNA was in great excess of the enzyme (which it was almost every time the enzyme was used) and it is suspected that more bands would have been

seen if a greater amount of Eco RI were used.

The DNA which was seen as a smear must by elimination be nuclear DNA. If either of the two DNAs were exposed to conditions which would tear it into small pieces during the isolation, it was the nuclear DNA, which gets ground in the Osterizer blender, exposed to DNase, shaken around through repeated centrifugations, stirred when pellets are resuspended and so forth. Thus, it is quite likely that the nuclear DNA would have been a smear on the gels, although chloroplast DNA could also have been a smear if it were sheared enough. If any of the nuclear DNA made it through the isolations whole then it may be the DNA seen at the tops of gels, since the native cpDNA will enter the gels and consequently could not be the DNA at the top of the gels (17).

The procedure presented in Materials and Methods would be very useful if it could be determined that the DNA isolated could indeed form the banding pattern expected of chloroplast DNA after digestion with Eco RI (1,18). This might occur if the DNA isolated were treated with an excess of restriction endonuclease. Under these conditions it is probable that the nuclear DNA would show up as a smear against which the chloroplast DNA would be visible.

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But this rough magic
I here abjure; and when I have required
Some heavenly music (which even now I do)
To work mine end upon their senses that
This airy charm is for, I'll break my staff,
Bury it certain fathoms in the earth,
And deeper than did ever plummet sound
I'll drown my book.

William Shakespeare,
The Tempest

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